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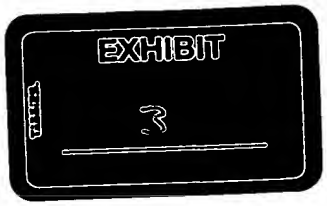
Collapsin: A Protein in Brain That Induces the Collapse and Paralysis of Neuronal Growth Cones



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Summary

Repulsive guidance cues can steer neuronal growth cones during development and prevent mature axons from regenerating. We have identified a 100 kd glycoprotein in the chick brain that is a good candidate for a repulsive cue. Since it induces the collapse and paralysis of neuronal growth cones in vitro, we have named it collapsin. It is effective at concentrations of ~10 pM. The C-terminal half of collapsin contains a single immunoglobulin-like domain and an additional highly basic region. The N-terminal half of collapsin shares significant homology with fasciclin IV, a growth cone guidance protein in grasshopper. Recombinant collapsin causes sensory ganglion growth cones to collapse but not retinal ganglion cell growth cones. We propose that collapsin could serve as a ligand that guides specific growth cones by a motility-inhibiting mechanism.

Introduction

The proper wiring together of the developing nervous system requires that each neuronal growth cone navigate accurately over a very long distance. Throughout the trajectory toward its target, a growth cone's behavior is controlled by cues in its surroundings. One class of relevant cues is thought to be specific, differentially localized adhesion molecules on cellular surfaces and in the extracellular matrix. A steadily growing number of cell adhesion molecules has been identified (reviewed by Jessell, 1988; Hortsch and Goodman, 1991). Some of them have been shown to be differentially localized in the developing nervous system and to support growth cone extension in vitro (e.g., Burns et al., 1991; Tanaka et al., 1991; Klar et al., 1992). The absence of specific adhesion molecules in vivo can be shown to perturb axon guidance (e.g., Harrelson and Goodman, 1988; Grenningloh et al., 1991; Hedgecock et al., 1990; Ishii et al., 1992; Leung-Hagesteijn et al., 1992). Another class of important guidance cues is likely to be chemoattractants that attract growth cones from a distance (reviewed by Tessier-Lavigne, 1992). However, their identification has proven to be difficult.

In contrast with these extension-promoting cues, there has recently been a growing appreciation that repulsive signals can direct growth cone extension. Perhaps the first hint of the importance of negative guidance cues was the finding by Tosney and Landmesser (1984) that outgrowing peripheral axons in chick embryos are channeled into a gap through the developing chondrocytes that form the pelvic girdle. Since then, repulsive cues have been implicated in growth cone guidance in several other systems. These include the formation of separate axon territories in culture when central and peripheral explants confront one another (Kapfhammer and Raper, 1987a, 1987b), the avoidance of posterior tectum by temporal retinal ganglion cell axons in the chick (Walter et al., 1987; Cox et al., 1990), the avoidance of posterior half-somites by spinal axons in the chick (Davies et al., 1990), the preference of temporal retinal ganglion cell growth cones to fasciculate on temporal as opposed to nasal retinal axons (Raper and Grunewald, 1990), and the avoidance of sensory axons by preganglionic sympathetic growth cones (Moorman and Hume, 1990).

Not only are repulsive cues thought to play a role in growth cone guidance during development, but they may also play an important role in determining whether damaged nerve fibers can regenerate in the mature central nervous system (CNS). Oligodendrocytes and CNS myelin express components on their surfaces that actively inhibit neurite outgrowth (Caroni and Schwab, 1988a, 1988b). Neutralization of these components has been reported to facilitate axon regeneration (Schnell and Schwab, 1990; Savio and Schwab, 1990). One intriguing possibility is that repulsive cues required for normal growth cone guidance during early development may interfere with axon regeneration later on.

Motile growth cones normally have a distinctive, spread morphology in culture conditions that support axon extension. Their morphology changes dramatically when they are confronted with repulsive cell surfaces. For example, a peripheral growth cone that makes contact with a retinal ganglion cell axon resorbs its thin lamellae and most of its filopodia. The growth cone collapses as these processes shrink toward the center of the growth cone. The collapsed growth cone is then temporarily paralyzed, failing to extend or retract lamellipodia and filopodia and failing to advance (Kapfhammer and Raper, 1987b). A similar sequence of events has been observed when growth cones meet other repulsive cell surfaces (Fawcett et al., 1989; Raper and Grunewald, 1990; Bandtlow et al., 1990).

We developed a simple in vitro assay for factors that induce growth cone collapse and paralysis in a fully permissive culture environment (Raper and Kapfhammer, 1990). It was shown using this assay that embryonic chick brain membranes contain a detergent-extractable activity that collapses the spread morphology of growth cones characteristic f motility. Video time-laps analysis showed that collapsed growth cones are paralyzed and their axons fail to advance. The same extracts had no comparable effect on motil fibroblasts, and membrane extracts prepared from liv r or fibroblasts had no significant collapsing activity on neurons. The collapsing effect was shown to b reversible and unaffect d by the substrate upon which growth con s extend. Since the activity is trypsin sensitive

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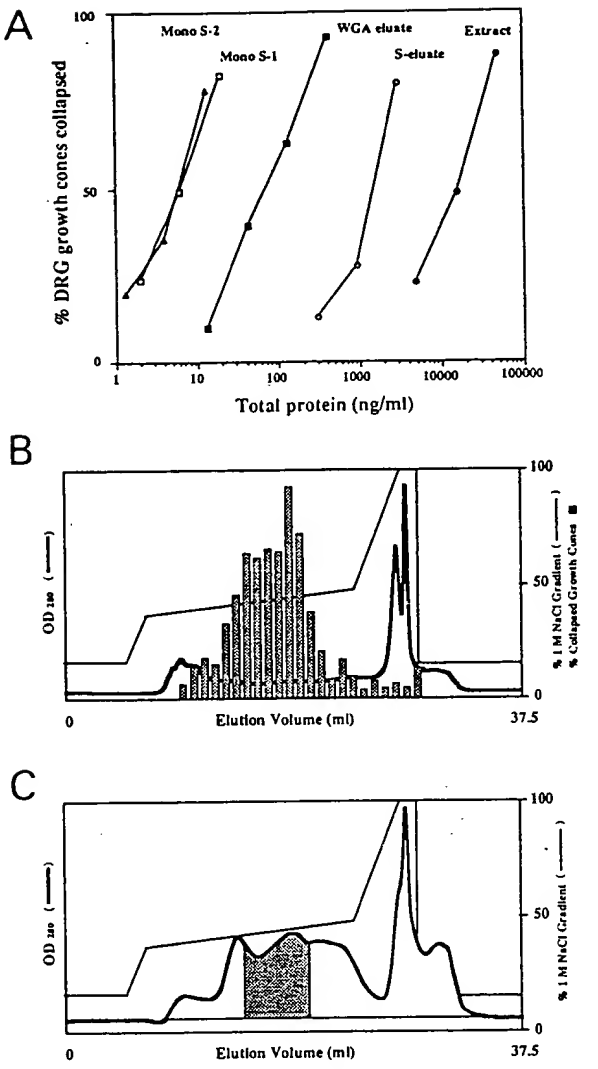

Figure 1. The Enrichment of a DRG Growth Cone–Collapsing Activity from Adult Chicken Brains

(A) The specific collapsing activity of material generated by each step in the purification was determined by the collapse assay. The percentage of collapsed growth cones is plotted against the concentration of added protein.

(B) The elution profile of the first Mono S run is presented. WGA-enriched material was loaded onto the Mono S column and eluted with the indicated salt gradient (thin line). The eluted protein was monitored by UV absorbance at 280 nm (bold line). The collapsing activity of each fraction is represented by the percentage of DRG growth cones that collapsed when 1 μl of eluate was added to 500 μl of culture medium (stippled bars).

(C) The elution profile of the second Mono S run is presented. The pooled active fractions from the first Mono S column were loaded onto the Mono S column for another round of elution. The stippled area indicates those fractions that caused the collapse of over 50% of DRG growth cones as assayed in (B).

and heat labile, the collapsing principle is likely to be a protein(s). This assay has been used by other groups to show that collapsing activities can be recovered from detergent-solubilized membrane xtracts of chick somites (Davies et al., 1990), posterior optic tectum (Cox et al., 1990), and CNS myelin (Bandtlow et al., 1993).

In this report we describe the partial purification of a growth cone–collapsing activity from adult chicken brains and the identification of a 100 kd glycoprotein that induces collapse. We have cloned and sequenced a cDNA encoding this protein and show that when transfected into COS cells, it directs the expression of a protein that collapses neuronal growth cones. We have therefore named this protein collapsin.

Results

Partial Purification of a Dorsal Root Ganglion Growth Cone–Collapsing Activity from Adult Chicken Brains

Our previous work showed that embryonic chick brain membranes contain a growth cone–collapsing activity (Raper and Kapfhammer, 1990). Cholate extracts from adult brain membranes are a more plentiful source of a similar activity. This activity, like that from embryonic day 10 (E10) chick brains, can be bound to and eluted from cation exchange and wheat germ agglutinin (WGA) affinity columns. These properties were used to enrich a growth cone–collapsing activity (Figure 1A). The detergent-solubilized membrane extract induces the collapse of 50% of DRG growth cones (specific collapsing activity) at a concentration of about 16 μg/ml. The extract was passed through a Q–Sepharose anion exchange column, and the wash through was loaded onto an S–Sepharose cation exchange column. Proteins eluted with a high salt buffer from the S–Sepharose column (S–eluate) had a specific collapsing activity of about 2 μg/ml. The S–eluate was then incubated with WGA-coupled agarose, and the WGA-bound activity was eluted with N-acetyl-D-glucosamine. The WGA–eluate had a specific collapsing activity of about 72 ng/ml. The WGA–eluate was bound to a Mono S cation exchange column, and the collapsing activity was eluted in the vicinity of 0.4 M with a NaCl salt gradient (Figure 1B). Fractions with a significant amount of collapsing activity were pooled, and the resulting pooled fraction had a specific collapsing activity of about 6 ng/ml. The pooled fraction was diluted with a low salt buffer, reapplied to the Mono S column, and again eluted with the same salt gradient (Figure 1C). The pooled active fraction from the second Mono S run had the same specific activity as that from the first Mono S run. Table 1 summarizes the purification procedure. Starting with membranes prepared from about 1500 adult chicken brains, 320 μg of protein was obtained. The overall enrichment of the collapsing activity was around 2600-fold with a yield of about 5%.

The Identification of a 100 kd Protein as the Collapsing Factor

To identify the protein responsible for inducing collapse, the eluted fractions from the last Mono S column were grouped into two pools: one containing all of the fractions with significant collapsing activity (stippled area in Figure 1C) and one containing all other fractions. The protein compositions of these two pools were compared in a SDS–polyacrylamide gel, along with those of the detergent-solubiliz d membrane extract and eluates from intermedi-

Table 1. Summary of the Partial Purification of Collapsin

Samples	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Extract	1.6×10^4	1.0×10^6	6.2×10^1	1	100
S-eluate	1.9×10^2	1.0×10^5	5.4×10^2	8.6	10
WGA	2.6×10^0	3.6×10^4	1.4×10^4	224	3.6
Mono S	3.2×10^{-1}	5.2×10^4	1.6×10^5	2624	5.2

Cholic acid–solubilized extracts from adult chicken brain membranes were used as the starting material (extract). The cumulative enrichment factors and yields are indicated for successive enrichment steps, including the eluate from S–Sepharose (S–eluate), the eluate from WGA-coupled agarose (WGA), and the pooled active fractions from the first Mono S run (Mono S). One unit of collapsing activity is defined as the amount of protein required to cause the collapse of 50% of DRG growth cones in 1 ml of medium.

ate steps in the purification (Figure 2). The active pool contained a major band around 100 kd and three other major bands around 200 kd. Of these, only the 100 kd band was present in the active pool but absent in the inactive pool. The 100 kd band also became increasingly prevalent as the collapsing activity was enriched. It was not visible in the starting or S–Sepharose-enriched material, was barely discernible in the WGA-enriched material, and became most prominent in the Mono S–enriched material, representing 10%–20% of the total staining intensity. When this material was run on a Superose-12 gel filtration column, the collapsing activity was eluted at an apparent molecular mass of around 120 kd (data not shown). If the 100 kd protein is assumed to constitute 10% of the most enriched pooled fraction with a specific activity of about 6 ng/ml, then it would cause 50% of DRG growth cones to collapse at about 10 pM. If the collapsing principle were instead a minor component of the same pool, it would probably need to be active below the 1 pM range. Taken together, these results indicated that the 100 kd protein was the most likely candidate for the collapsing activity.

Partial Amino Acid Sequence of Collapsin

To obtain partial amino acid sequences from the 100 kd protein, the proteins in the most enriched pool were separated on an SDS–polyacrylamide gel, blotted onto a nitrocellulose membrane, and stained with Ponceau S. Approximately 10 μg of the 100 kd protein was cut out of the blot and digested with trypsin. The tryptic peptide fragments were separated by reverse phase high pressure liquid chromatography. Four major peptide peaks were recovered, and their amino acid sequences were determined by automated Edman degradation. Each peak yielded a single peptide sequence: XXPNYQWVPYQGR, XXLEVIDTDHLEELLHK, XPIVYGVFT, and XLPDEVITFA, where X indicates an unidentified residue. A search of protein data bases using the BLAST program (Altschul et al., 1990) showed that the four peptide sequences do not match any previously known protein sequences.

Isolation of a cDNA Clone Containing the Four Partial Amino Acid Sequence

To identify a collapsin cDNA by the polymerase chain reaction (PCR), four degenerate oligonucleotides corresponding to the tryptic peptide sequences, along with their complementary sequences, were designed and synthesized. These degenerate oligonucleotides were used as primers to amplify cDNAs prepared from the total RNA of E20 chick brains. A 1.1 kb PCR fragment derived from 2 of the 4 oligonucleotides hybridized with two oligonucleotide probes that were not used as primers in its generation. This 1.1 kb PCR fragment was isolated and subcloned into a plasmid vector. Analysis of the nucleotide and the deduced amino acid sequences of the 1.1 kb PCR fragment indicated that it encodes an incomplete open reading frame containing all four tryptic peptide sequences. This 1.1 kb fragment was then used to probe a λgt10 adult chicken brain cDNA library. The four largest inserts identified in this way were sequenced. Since none of them contained a complete open reading frame, another round of screening was done with oligonucleotides corresponding to the 5′ most of the known sequences. This screen resulted in the identification of a single 3.2 kb cDNA insert encoding the entire collapsin sequence.

Nucleotide and Deduced Amino Acid Sequences of Collapsin

The 3.2 kb insert contains a single open reading frame

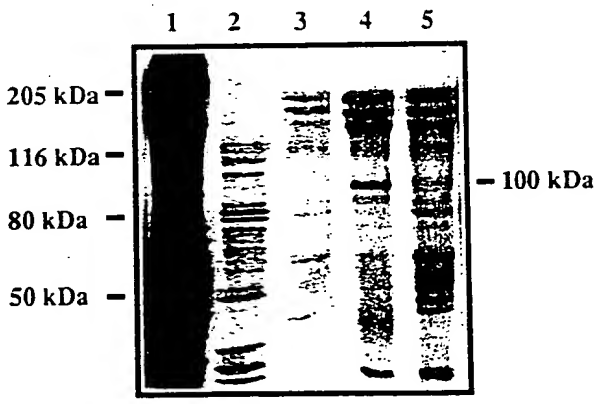

Figure 2. Identification of a 100 kd Candidate for the Collapsing Activity by SDS–Polyacrylamide Gel Electrophoresis

Proteins from the detergent-solubilized membrane extract (80 μg, lane 1), the S–eluate (20 μg, lane 2), the WGA eluate (15 μg, lane 3), the pooled active fractions of the second Mono S run indicated by the stippled area in Figure 1C (15 μg, lane 4), and a pool containing all other (inactive) fractions of the second Mono S (15 μg, lane 5) were run in a 7.5% SDS–polyacrylamide gel and stained with Coomassie blue. Molecular masses are indicated on the left. A 100 kd band is prominent in the pooled active fractions and is nearly absent in the inactive fractions.

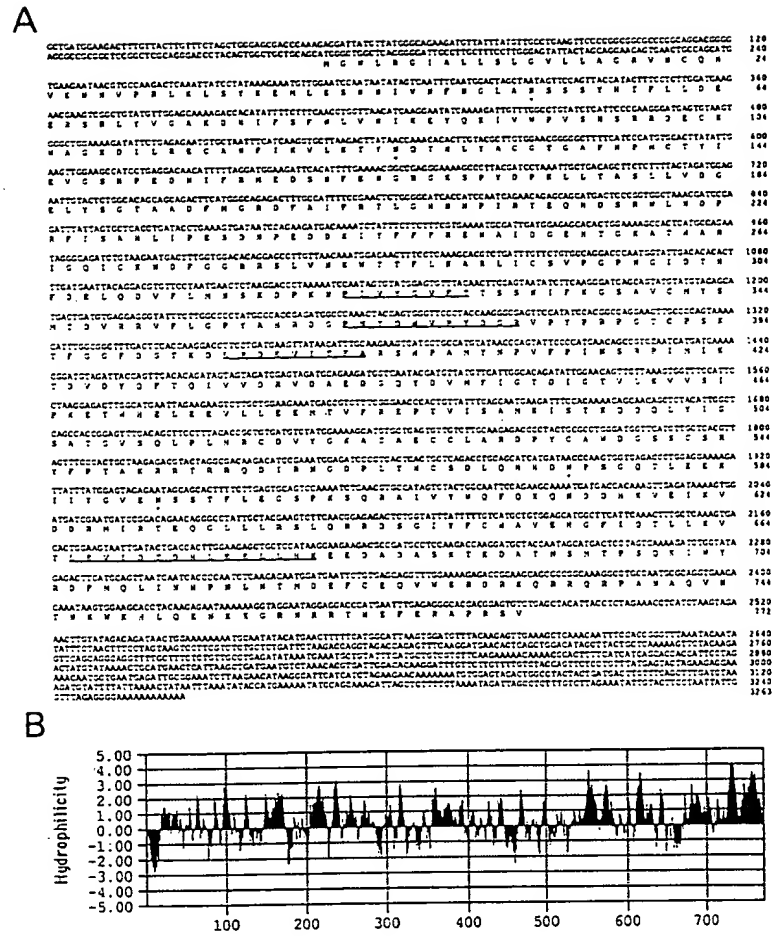

Figure 3. The Nucleotide and Predicted Amino Acid Sequences of Collapsin

(A) The positions of nucleotide and amino acid residues are given at the right. The four tryptic peptide sequences obtained by microsequencing the native protein are underlined. Four potential N-linked glycosylation sites are marked by asterisks.

(B) A hydropathy plot for the predicted collapsin protein was generated by the method of Kyte and Doolittle (1982). The values of hydrophobicity are plotted against the positions of the amino acid residues. Negative values indicate increasing hydrophobicity.

starting with an ATG start codon at nucleotide 168 and ending with a TGA stop codon at nucleotide 2483 (Figure 3A). The open reading frame predicts a protein of 772 amino acids with a molecular weight of 88,811 daltons and a pI of 7.65. The start codon at nucleotide 168 is a strong translation initiator by sequence context criteria (Kozak, 1989) and is preceded with an in-frame stop codon at nucleotide 3. Another in-frame methionine codon was found upstream at nucleotide 60. Whether this codon can serve as an initiation site for translation is not known. This codon is not required for the expression of active collapsin in COS-7 cells (see below). The stop codon at 2483 is followed by a 780 nt untranslated sequence ending with a poly(A) tail that is not preceded by the consensus polyadenylation signal AATAAA.

Hydropathy analysis of the predicted amino acid sequence (Kyte and Doolittle, 1982; Figure 3B) revealed a prominent stretch of hydrophobic sequence at the N-terminus of collapsin that could function as a signal peptide for protein secretion (von Heijne, 1985). The signal peptide cleavage sites were predicted to be at residues Ala-17 or Cys-22 (von Heijne, 1986). The hydropathy analysis did not show any other long stretches of hydrophobic residues that could serve as a potential transmembrane-spanning domain. The deduced protein sequence contains four potential sites for N-linked glycosylation (indicated by asterisks in Figure 3A). The apparent discrepancy between the predicted molecular mass of 88 kd and the native molecular mass of 100 kd may be explained by glycosylation of the core protein at these sites. The four tryptic peptide sequences obtained from amino acid sequencing are underlined in Figure 3A.

Structural Features of Collapsin

A protein data base search using the BLAST program revealed that collapsin is a novel protein with several prominent structural features. First, the N-terminal portion of collapsin between residues 100 and 540 shares significant homology with residues 94–520 of fasciclin IV (Kolodkin et al., 1992; Figure 4A). Close comparison of the two sequences shows that 12 of the 14 cysteines within this region of fasciclin IV are conserved in collapsin. There is an approximately 35% identity between the aligned sequences over a 400 amino acid stretch. Another domain is defined in collapsin by two cysteine residues (Cys-598 and Cys-626). The closest match in the regions immediately surrounding each of these two cysteines is with the β chain of the T cell receptor (Kimura et al., 1987; Figure 4B); however, the sequence between the cysteines is significantly shorter than the T cell receptor sequence. The spacing between the cysteines and the sequences around

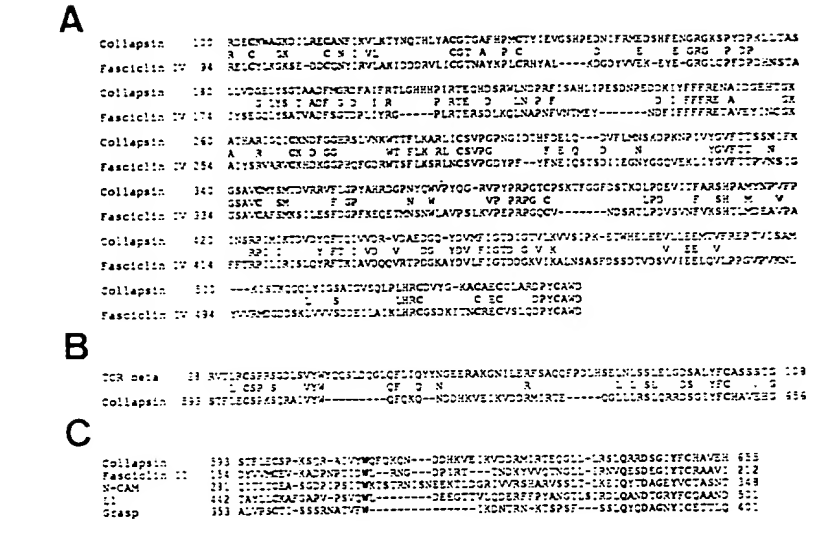

Figure 4. Homologies of Collapsin to Fasciclin IV and Selected Immunoglobulin Superfamily Members

(A) An alignment comparing the amino acid sequences of collapsin and fasciclin IV was generated by the Align module of the Pearson sequence analysis package (Myers and Miller, 1988). Identical residues are indicated between the aligned sequences.

(B) The immunoglobulin-like domain of collapsin is aligned with that of the β chain of the human T cell receptor. Identical residues are indicated between the aligned sequences, and cysteine residues are shown in bold.

(C) The immunoglobulin-like domain of collapsin is aligned with selected C2 domains from several neuronal cell adhesion molecules. Only those amino acids conserved in all members of the group are shown in bold.

them fit the general consensus for an immunoglobulin-like domain of the C2 type (Williams and Barclay, 1988; Hunkapiller and Hood, 1989). The closest match within this group is with fasciclin II, an adhesion molecule that promotes the fasciculation of specific axons in the insect CNS (Harrelson and Goodman, 1988; Grenningloh et al., 1991). Figure 4C shows alignments of the immunoglobulin-like domain in collapsin with selected C2 domains from several neuronal cell adhesion molecules (Hemperly et al., 1986; Moos et al., 1988; Burns et al., 1991). There are 7 amino acids that are conserved between collapsin and all of the others and several additional matches between residues in collapsin and corresponding residues in individual members of the group.

Functional Expression of Collapsin cDNA in COS-7 Cells

To determine whether the cloned collapsin cDNA encodes a protein with collapsing activity, we subcloned the collapsin cDNA into a mammalian expression vector and transfected the resultant plasmid into COS-7 cells. Medium conditioned for 48 hr by cells transfected with collapsin cDNA induced the collapse of DRG growth cones in a dose-responsive manner, whereas medium conditioned by mock-transfected cells had no apparent collapsing activity (Figure 5A). Medium conditioned by cells transfected with the same vector containing a cDNA encoding the homophilic adhesion molecule DM-GRASP (Burns et al., 1991) also did not induce the collapse of DRG growth cones (data not shown). Although the majority of the recombinant collapsing activity was present in the conditioned medium, it was also recovered from detergent-solubilized extracts of collapsin-transfected cells (Figure 5B). Detergent extracts of the mock-transfected and GRASP-transfected cells had no collapsing activity. Th morphologies of DRG growth cones that have been induced to collapse by recombinant collapsin are indistinguishable from those exposed to the native brain-derived collapsing activity (Figure 6).

Collapsin Does Not Induce the Collapse of Retinal Growth Cones

To determine whether recombinant collapsin is specifically targeted to some neurons and not others, its ability

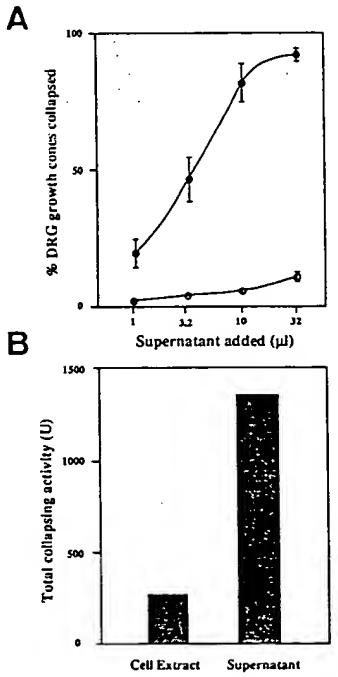

Figure 5. The Expression of Collapsin cDNA in COS-7 Cells

(A) The collapsing activity of conditioned medium from cultures of COS cells transfected with collapsin cDNA (closed circles) is compared with that from mock-transfected cells (open circles). The percentage of collapsed DRG growth cones is plotted against the volume of COS cell supernatant added to 500 μl of culture medium displayed on a logarithmic scale. SEM is indicated.

(B) A comparison of the total collapsing activity recovered from medium conditioned by transfected cells and the activity recovered from detergent extracts of the transfected cells themselves. One unit of collapsing activity is defined as the amount of protein required to induce the collapse of 50% of DRG growth cones in 1 ml of medium.

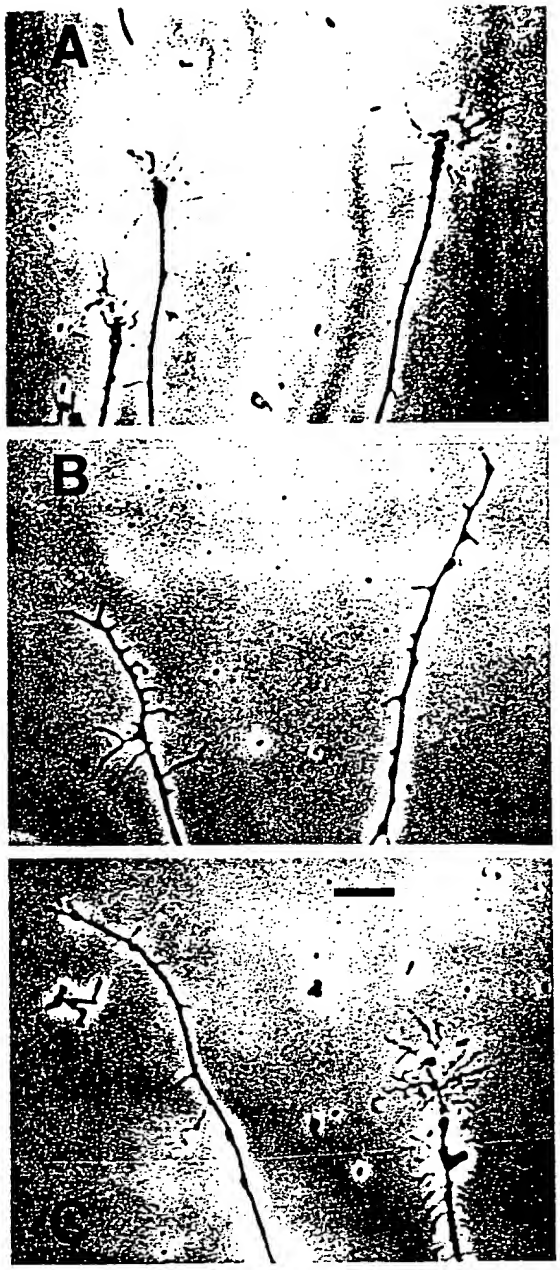

Figure 6. A Comparison of the Effects of Native and Recombinant Collapsin on DRG Growth Cones

The morphologies of DRG growth cones treated with conditioned medium from mock-transfected cells (A), conditioned medium from cells transfected with collapsin cDNA (B), and WGA–eluate from the enrichment of the brain-derived collapsing activity (C). Calibration: 20 μm.

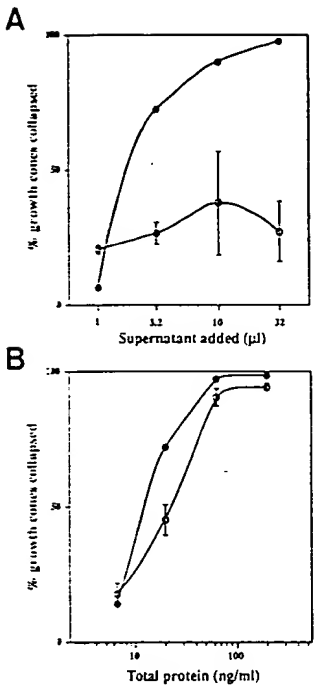

Figure 7. Recombinant Collapsin Does Not Induce the Collapse of Retinal Ganglion Cell Growth Cones

(A) The relative responsiveness of DRG growth cones (closed circles) and retinal ganglion cell growth cones (open circles) to conditioned medium from COS cells transfected with collapsin cDNA. The percentage of collapsed growth cones is plotted against the volume of COS cell supernatant added to 500 μl of culture medium displayed on a logarithmic scale. SEM is indicated for the retinal growth cones.

(B) The relative responsiveness of DRG growth cones (closed circles) and retinal ganglion cell growth cones (open circles) to the WGA–eluate of partially enriched native collapsin. The percentage of collapsed growth cones is plotted against the concentration of added protein displayed on a logarithmic scale.

to induce the collapse of DRG and retinal ganglion cell growth cones was compared. Recombinant collapsin causes DRG growth cones to collapse in a dose-responsive manner at concentrations that have little effect on retinal ganglion cell growth cones (Figure 7A). Neither temporal nor nasal retinal growth cones collapse with the addition of recombinant collapsin (data not shown). In contrast, both DRG and retinal growth cones collapse in the presence of WGA–eluate from adult chicken brains (Figure 7B).

Northern Analysis of the Expression of Collapsin

To determine the size and tissue distribution of collapsin mRNA, we carried out Northern blot hybridizations on mRNA prepared from various E15 chick tissues. As shown in Figure 8A, the collapsin cDNA probe hybridized with a major band of about 7.2 kb and a minor band of about 4.0 kb. Both transcripts are large enough to contain the 3.2 kb collapsin cDNA. The collapsin gene was expressed at relatively high levels in brain and muscle, moderate levels in lung, bursa, and heart and was virtually absent in liver. The levels of collapsin mRNA in brain as compared with liver are consistent with our previous finding that brain membranes have collapsing activity while liver membranes do not (Raper and Kapfhammer, 1990). Collapsin was found to be expressed at roughly comparable levels in messenger RNA prepared from whole brains of E4, E6, E10, and E20 embryos (Figure 8B).

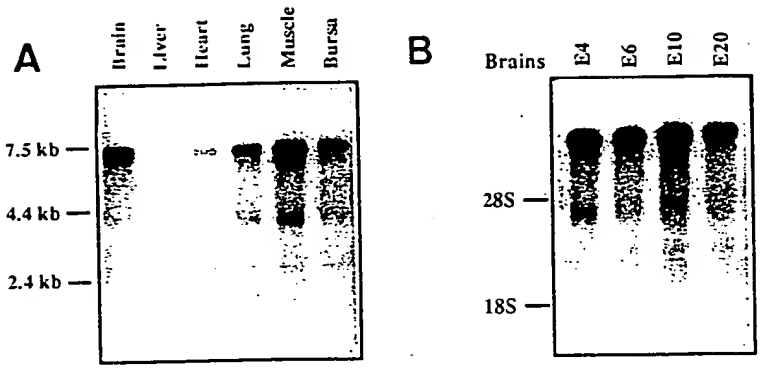

Figure 8. Northern Analysis of Collapsin Expression

(A) E15 chick tissues were used to make purified mRNA. mRNA (2 μg) was loaded into each lane and probed with the full coding sequence of collapsin. The positions of RNA standards are indicated to the left.

(B) Purified mRNA was prepared from E4, E6, E10, and just hatching E20 brains. mRNA (2 μg) was loaded into each lane and probed with the full coding sequence of collapsin. The positions of 18S and 28S ribosomal RNA are indicated.

Discussion

Several lines of evidence indicate that we have identified a growth cone–collapsing protein and cloned its corresponding cDNA. Collapsin was coenriched with collapsing activity through several purification steps. It is present in the active fractions of our most enriched preparation but is absent in the complementary inactive fractions. Most importantly, expression of the collapsin cDNA in COS-7 cells produces a DRG growth cone–collapsing activity that is not produced by mock-transfected cells.

There are several interesting features to the collapsin sequence. First, collapsin contains a signal peptide sequence but no apparent transmembrane-spanning region. Moreover, recombinant collapsin is secreted from COS cells transfected with collapsin cDNA. This suggests that collapsin is a secreted protein. However, collapsin contains a very basic region near its C-terminal end. This basic tail may serve to bind collapsin to negative charges displayed by cellular membranes or extracellular matrices, and this may explain why collapsin is found in the brain membrane preparation that was used as the starting material for our purification. This hypothesis is also consistent with our earlier finding that approximately one-half of the collapsing activity in brain membranes can be solubilized with high salt buffers (Raper and Kapfhammer, 1990). Since collapsin is likely to be secreted and may also bind to cell surfaces or to extracellular matrices, it could act either as a soluble repellant of growth cones or participate in contact-mediated interactions. A soluble repellent would be without precedent, except for the recent report by Pini (1993) of an activity secreted by septal tissue that repulses olfactory tract axons in culture.

There is a strong resemblance between the N-terminal half of collapsin and the extracellular portion of a transmembrane protein in the grasshopper, fasciclin IV (Kolodkin et al., 1992). Fasciclin IV is expressed on some but not all axon pathways in the embryonic grasshopper CNS and on circumferential bands of epithelial cells in the limb buds. Antibodies to fasciclin IV perturb axonal extension from identified pioneer neurons in the limb bud. Th Ti1 neurons normally grow from the periphery into th ventral nerve cord along a stereotyped pathway. They make an orthogonal approach to a stripe of fasciclin IV–expressing cells, and when they reach them, they turn and extend parallel to the stripe for some distance. Ti1 growth cones extending in the presence of antibodies to fasciclin IV often fail to turn at the stripe, or after turning, run in an uncharacteristically loose fascicle along the stripe. The similarity between collapsin and fasciclin IV raises the possibility that fasciclin IV induces the turn of the Ti1 pioneer growth cones by a repulsive mechanism.

Collapsin also contains a single C2 type immunoglobulin-like domain. It is worth noting that immunoglobulin-like domains commonly occur in adhesion molecules such as L1/G4, DM-GRASP, TAG-1, and the N-CAM that promote growth cone extension (Hemperly et al., 1986; Moos et al., 1988; Furley et al., 1990; Burns et al., 1991). Since collapsin inhibits motility and extension, the presence of immunoglobulin-like domains seems not to be diagnostic for adhesive or growth-promoting functions. By comparison with the known functions of other proteins with immunoglobulin-like domains, this region may contribute to dimerization with itself or its association with other proteins containing immunoglobulin loops (reviewed by Williams and Barclay, 1988).

Perhaps our most striking observation is that recombinant collapsin exhibits an apparent specificity toward a subset of neuronal growth cones. It induces the collapse of DRG growth cones at concentrations that have little effect on retinal ganglion cell growth cones. In contrast, collapsin-containing WGA–eluate from adult chicken brains induces the collapse of both DRG and retinal growth cones. It is possible that recombinant collapsin, because it is produced in a foreign cell line, is proteolytically processed or glycosylated in a way that does not preserve all aspects of its native activity. Another possibility is that like recombinant collapsin, native collapsin affects DRG but not retinal growth cones, and the WGA–eluate contains an independent collapsing activity that affects retinal growth cones. Given the presence of two messages that hybridize with collapsin cDNA, it is possible that differentially spliced versions of collapsin affect different populations of growth cones. Alternatively, there may be a family of bioch mically related collapsing molecules that have differing target specificities.

Collapsin has a specific activity in the 10 pM range. This high potency is comparable to that of many growth and

differentiation factors, raising th possibility that collapsin could act through a ligand–receptor-mediated signal transduction mechanism. Perhaps collapsin induces collapse through a G protein–coupled r c ptor, since pertussis toxin decreases the sensitivity of growth cones to a relatively crude preparation of collapsing factor prepared by our methods (Igarashi et al., 1993). However, this modest loss of sensitivity may reflect some other change in growth cones that makes them more difficult to collapse, rather than a blockade of the transduction pathway. A myelin-derived activity that inhibits growth cone extension has also been reported to act through a pertussis toxin–sensitive pathway (Igarashi et al., 1993) and an elevation of intracellular Ca^{2+} (Bandtlow et al., 1993). In contrast, Ivins et al. (1991) found no significant changes in intracellular Ca^{2+} levels when partially enriched preparations of native collapsin were applied to DRG growth cones.

Partially purified native collapsin appears to induce a dramatic loss of polymerized actin at the leading edge of growth cones (Fan et al., 1993). Collapse occurs as the actin meshwork that supports growth cone morphology is lost. Paralysis is likely to be a direct consequence of failed actin polymerization since it is the polymerization of actin that is thought to drive the extension of the leading edge of an advancing growth cone (Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986; Theriot et al., 1992). Collapsin could theoretically steer a growth cone if its effects on actin polymerization and motility are confined to only that region of the growth cone exposed to a high concentration of collapsin. Alternatively, the whole growth cone will be affected if a localized exposure causes actin depolymerization throughout the growth cone. Then collapsin could act as a guidance cue by eliminating inappropriate growth cones from forbidden territories.

The biological role of collapsin in the nervous system is unknown. The ability of collapsin to induce the collapse of specific growth cones, its structural homology to the growth cone guidance molecule fasciclin IV, and its presence in the very early embryonic chick brain all make collapsin a good candidate for a role in growth cone guidance during the development of the chick nervous system. Its presence in the adult CNS raises the possibility that it could interfere with the regeneration of damaged axonal processes. The presence of collapsin in many nonneuronal tissues may indicate either that it plays a role in axon guidance in the periphery or that it has broader functions.

Experimental Procedures

Membrane Preparation from Adult Chick Brains

Adult chicken brain membranes were prepared as described previously for embryonic chick brain membranes (Raper and Kapfhammer, 1990) with some modifications. Brains flash frozen in liquid nitrogen (Pelfreeze) were thawed in Hank's balanced salt solution with additional 10 mM HEPES (pH 7.4), 1 mM EDTA, 50 μM leupeptin, and 10 μM para-nitrophenylguanidino benzoate. Ten milliliters of the buffer was used to thaw each brain. Thawed brains were disrupted for 30 s in a table-top blender set at a low speed. The crude membranes were pelleted at 10,000 × g and washed three times with HEPES and EDTA in Hank's balanced salt solution. The washed and pelleted crude membranes from each brain equivalent were diluted with 5 ml of 2.25 M sucrose in phosphate-buffered saline (PBS) and blended for another 30 s at a low speed. Each 100 ml of this mixture was layered on top of 25 ml of 2.25 M sucrose in PBS. An additional 50 ml of 0.8 M sucrose in PBS was layered on top of the suspended membranes. After centrifugation at 12,000 × g for 75 min at 4°C, the membranes at the interface between the middle and top layers were collected, washed three times in 0.5 × PBS with 10 mM Tris (pH 7.4) and frozen at −70°C until needed.

Protein Purification

Frozen membranes were thawed in 0.5 × PBS, 3% cholic acid, and 10 mM Tris (pH 7.4) at a ratio of 1 brain equivalent per 5 ml. The thawed membranes were homogenized in a Dounce homogenizer and centrifuged at 100,000 × g for 1 hr at 4°C. The supernatant was collected and passed through a Q–Sepharose (Pharmacia) anion exchange column that was preequilibrated with 1 × PBS, 5 mM Tris (pH 7.4) and 0.1% cholic acid. One milliliter of resin was used for each 4 brain equivalents of supernatant. The flow through was then loaded onto a S–Sepharose (Pharmacia) cation-exchange column preequilibrated with 1 × PBS in 5 mM Tris (pH 7.4) and 0.1% cholic acid at a ratio of 4 brain equivalents per ml of resin. The column was washed with the equilibration buffer and eluted with 3 column volumes of the same buffer containing an additional 0.5 M NaCl. The eluate (S–eluate) was then incubated with WGA-coupled agarose (Vector) at a ratio of up to 80 brain equivalents per 0.5 ml resin. After a minimum of 2 hr incubation at 4°C, the agarose beads were washed with 20 mM Na_2HPO_4 (pH 7.2), 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, and 5% glycerol, and eluted with 4 column volumes of this wash buffer augmented with 0.5 M N-acetyl-D-glucosamine.

The WGA–eluate was loaded onto a Mono S cation-exchange column (HR5/5, Pharmacia) equilibrated with 90% buffer A (20 mM Na_2HPO_4 [pH 7.2], 1 mM EDTA, 0.1% CHAPS, and 5% glycerol) and 10% buffer B (A + 1 M NaCl) at a flow rate of 0.5 ml/min. The elution profile was: 90% A + 10% B for 20 min; 90%–67% A + 10%–33% B over 3 min; 67%–55% A + 33%–45% B over 36 min; 55%–0% A + 45%–100% B over 8 min. Fractions (1 ml each) were collected and 1 μl of each fraction was added to 500 μl of medium in an assay to determine its collapsing activity. Those fractions that induced over 50% of DRG growth cones to collapse were pooled, diluted with an equal volume of buffer A, and rerun on the Mono S column using the same flow rate and elution profile.

Peptide Sequence Analysis

The material in the pooled active fractions from the last Mono S run were concentrated in a Centricon 30 (Amicon) and run on a 7.5% SDS–polyacrylamide gel, blotted onto a nitrocellulose membrane (Bio-Rad), and stained with 0.5% Ponceau S in 1% acetic acid. The 100 kd band was cut out, digested in situ with trypsin (Fernandez et al., 1992), and separated by reverse phase high pressure liquid chromatography. Four tryptic fragments were chosen for automated protein sequence analysis with an Applied Biosystems Model 475A Protein Sequencer in the gas phase mode. The tryptic digestions and microsequencing were performed by the Protein Micro-chemistry Facility of the Wistar Institute under the direction of D. Speicher.

Neural Explants and Cell Culture

DRG and retinal explants were prepared as previously described (Chang et al., 1987; Fan et al., 1993). In brief, DRGs removed from E7 chick embryos were cut into halves or quarters, plated onto 9 mm glass coverslips coated with laminin, and cultured in a standard 48-well microplate (Costar) for 18–24 hr in a defined medium supplemented with bovine pituitary extract (Tsao et al., 1982). Small bits of E6 retinal tissue were explanted similarly. COS-7 cells (kindly provided by Dr. Burns) were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Collapse Assay

The procedure for the collapse assay was essentially the same as previously described (Raper and Kapfhammer, 1990). In brief, small aliquots of solutions to be tested for collapsing activity were added to DRG explants. The added material was gently mixed into the culture medium, the cultures were incubated at 37°C in 5% CO_2 for 1 hr, and then fixed with 4% paraformaldehyde in PBS containing 10% sucrose.

The fixed preparations were then scored for the number of neurite tips without lamellipodia or filopodia (collapsed) and the number of normal growth cones. The data are presented as the percentage of collapsed growth cones as a proportion of the total number of growth cones counted. Before each bioassay, the protein concentration of each sample tested was determined according to the method of Bradford (1976). The cholic acid–solubilized membrane extracts were dialyzed against PBS overnight at 4°C before being used for the bioassay. All other samples were used directly without further treatment.

PCRs

The four peptide sequences obtained by microsequencing were used to synthesize the following degenerate oligonucleotides and their complementary sequences:

1: 5′-CC(CT)TG(AG)TA(ACGT)GG(ACGT)ACCCA(CT)TG(AG)TA(AG)TT-3′.
2: 5′-TG(AG)TC(ACGT)GT(AG)TC(AGT)AT(ACGT)AC(CT)TC-3′.
3: 5′-GT(AG)AA(ACGT)AC(ACGT)CC(AG)TA(ACGT)AC(AGT)AT-3′.
4: 5′-GT(AGT)AT(ACGT)AC(CT)TC(AG)TC(ACGT)GG-3′.

The GeneAmp RNA PCR Kit (Perkin Elmer Cetus) was used for cDNA synthesis and subsequent PCRs following the manufacturer's instructions. In brief, single-strand cDNA was synthesized from E20 chick brain total RNA using Moloney murine leukemia virus reverse transcriptase and random primers. Different combinations of the degenerate oligonucleotide pairs (12 possible pairs generating 6 possible products from the 8 oligonucleotides) were then added to the cDNA and amplified with Taq DNA polymerase. The protocol for the PCR was: an initial 2 min at 95°C followed by 5 cycles of 95°C for 40 s, 44°C for 1 min, 72°C for 1 min; 5 cycles of 95°C for 40 s, 42°C for 1 min, 72°C for 1 min; 5 cycles of 95°C for 40 s, 40°C for 1 min, 72°C for 1 min; 35 cycles of 95°C for 40 s, 38°C for 1 min, 72°C for 1 min; and a final incubation at 72°C for 5 min. The PCR products from the 24 reactions were separated into four groups. Each group contained the PCR products generated by all combinations of 3 of the 4 primers and their complements. The PCR products from each group were fractionated on 1.5% agarose gels, transferred to nylon membranes (Stratagene), and hybridized with the omitted oligonucleotide probe end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook et al., 1989). A 1.1 kb PCR fragment derived from the oligonucleotide pair of number 2 and the complementary sequence of number 3 was identified that hybridized with the radiolabeled oligonucleotide probes numbers 1 and 3. The 1.1 kb PCR fragment was isolated and subcloned into the vector pCR-II using the TA cloning kit (Invitrogen).

Isolation of cDNA Clones

After verification that the 1.1 kb fragment contained sequences coding for all of the four known peptides, it was used to generate probe by random priming (Feinberg and Vogelstein, 1983). The probe was used to screen an adult chicken brain cDNA library in bacteriophage λgt10 (Clontech). Forty positive clones were identified from 2×10^6 phage plaques, and 20 of them were purified by an additional three rounds of screening. The insert size of the purified clones was estimated by PCR using phage-derived primers (Clontech) to amplify the insert. The four largest inserts (each about 2 kb) were subcloned into the plasmid Bluescript II (Stratagene) and sequenced. None of the four inserts contained a complete open reading frame. Therefore, two 28 base oligonucleotides based upon the 5′ most of the available cDNA sequences were synthesized, end labeled, and used to screen an additional 2×10^6 phage plaques. Five positive clones were identified and purified, and their inserts were subcloned into Bluescript II. The plasmid with the largest insert (3.2 kb) was chosen for DNA sequence analysis.

DNA Sequence Analysis

Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using double-stranded DNA as template and T7 DNA polymerase (Sequenase, US Biochemicals). Both strands of the cDNA insert were sequenced using synthetic oligonucleotides as primers. The MacVector (IBI) software package was used to analyze nucleic and amino acid sequences. Searches for related sequences were done through the BLAST network service provided by the National Center for Biotechnology Information.

Expression of Collapsin cDNA in COS-7 Cells

A unique SacII restriction site 37 bases upstream of the ATG initiation site and a unique XbaI site 11 bp downstream of the TGA stop codon were used to cut out a DNA fragment containing the full coding region of the collapsin gene. This fragment was subcloned to the NotI–XbaI site of the mammalian expression vector pRC/RSV (Invitrogen) using two partially complementary oligonucleotides as an adaptor. Resultant plasmid DNA (16 μg) was transfected into COS-7 cells by calcium phosphate precipitation in 60 mm dishes (Sambrook et al., 1989). After 16 hr in culture, the cells were washed with PBS, given fresh medium, and cultured for an additional 48 hr. The conditioned medium was used directly in a collapse assay after centrifugation at 5000 × g for 10 min to remove debris. The transfected cells in one 60 mm dish were harvested with a rubber policeman, washed with PBS, solubilized in 200 μl of 2% cholic acid in PBS for 5 min at 4°C, and centrifuged at 14,000 × g for 10 min. The resulting supernatant was collected and dialyzed against PBS at 4°C overnight before testing its activity in the collapse assay.

Northern Blot Analysis

mRNA was isolated from various tissues using the micro Fast Track mRNA isolation kit (Invitrogen) following the manufacturer's instructions. Two micrograms of mRNA from each tissue was fractionated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane (Biorad). The filter was hybridized overnight at 42°C in 50% formamide, 6 × SSPE, 2 × Denhardt's solution, 100 μg/ml salmon sperm DNA, and 1×10^6 cpm/ml probe (Sambrook et al., 1989). The SacII–XbaI fragment encoding the entire collapsin coding sequence was labeled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). Following hybridization, the filter was washed to a final stringency of 0.2 × SSC and 0.1% SDS at 65°C. The washed filter was used to expose Kodak XAR-5 film with an intensifier at −70°C for 48 hr. The sizes of the mRNA transcripts were estimated by comparison with a series of RNA standards (GIBCO).

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